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**13. ABSTRACT** *(Maximum 200 Words)*

We propose to establish an approach by which tumor cells are eradicated through selective induction of $CD8^+$ specific for a cellular protein that is expressed in many breast and ovarian cancers. Our model system is the class I MHC molecule HLA-A2.1 and the HER-2/neu protein. HLA-A2.1 is present in approximately 50% of Caucasians and African-Americans, and HER-2/neu is overexpressed in approximately 30% of adenocarcinomas including breast cancer. A peptide derived from HER-2/neu (HN654-662) has been shown to bind HLA-A2.1 and stimulate cytotoxic T lymphocytes (CTL) that lyse primary tumors from breast or ovarian cancer. The peptide has poor immunogenicity due to poor binding to HLA-A2.1 ($T_m$ = 36.6 °C). We are trying to improve the binding affinity by making substitution in the peptide sequence to make it effective therapeutic agent. The crystallographic structure of HN654-662 co-crystallized with HLA-A2.1 shows that the center of the peptide does not assume one specific conformation and does not make stabilizing contacts with the peptide binding cleft.. The altering of the primary anchor residues did not improve the binding significantly. Out of many variants, only V5L, is relatively more stable with higher Tm (Tm=45.8 °C). The crystallographic studies of HLA-A2.1 plus HN654-662 variant (I2L/V5L/L9V) shows that substituted Leu at fifth position point away from the MHC molecule. It seem that substitution of anchor residues makes Leu to point away. We expect that in V5L variant, the substituted Leu will make stabilizing contacts.

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# INTRODUCTION

Class I major histocompatibility molecules (MHC) bind short peptides derived from proteins synthesized within the cell. Cytotoxic T Lymphocytes (CTL) are activated when their T cell receptors (TCR) recognizes a complex of peptide and class I MHC molecule and lyse the cell expressing the complex. This process eliminates some tumor cells expressing new or altered proteins. However, not all tumors are recognized efficiently. One reason hypothesized is poor binding of those peptides to class I MHC molecules. Studies have shown that increasing the binding affinity of a peptide can improve its immunogenicity. Many peptides derived from the proto-oncogene HER-2/neu have been shown to be recognized by cytotoxic T cells derived from HLA-A2+ patients with breast cancer and other adenocarcinomas. One of the peptides, GP2 (HN654-662) binds very poorly even though it is predicted to bind well based upon the presence of the correct HLA-A2.1 peptide-binding motif. Creating peptides with greater affinity for HLA-A2.1 that are still recognized by HN654-662 specific CTL will increase CTL efficiency against wild type HER-2/neu HLA-A2.1 complexes and will result in complete eradication of the cancer from the patient. We propose to identify variant peptides that increase the affinity of class I MHC/peptide complex for HER-2/neu specific TCR.

## ANNUAL SUMMARY

**Specific Aim: Develop HN-654-662 variant peptides with improved affinity for HLA-A2.1**

**1.1 Determine the co-crystal structure of HLA-A2.1 complexed with wild type HN654-662 peptide.**

**Rationale**: The structures of a number of single peptide/MHC class I co-crystal structures are now known. These structures unfortunately have not allowed us to predict how any single peptide will bind. Much more structural information is required to begin to explain the multitude of mutational and kinetic data already present. The experiments described here seek to address part of this issue. What property of the HN654-662 peptide makes it a poor ligand for HLA-A2.1? These data will guide us in designing second generation epitopes.

**Approach**: Using synthetic peptides and in vitro folding of HLA-A2.1, sufficient quantities of complex are easily obtained for crystallization. The data will be collected on an R-AXIS II area detector system and the data processed with the program DENZO. The structures will be determined by molecular replacement using the program suite AMoRe and refined by iterative cycles of manual rebuilding using the graphical program O, computational refinement with X-PLOR and if the molecule crystallizes with more than one molecule per asymmetric unit, averaging using the RAVE suite. The resulting structure will be directly compared with crystal structure of HLA-A2.1/peptide complex for which the peptide has high affinity (for example: calreticulin signal sequence, HIV matrix peptide)

**Work done:** In order to understand why HN654-662 binds poorly to A2.1, we determined the crystallographic structure of A2.1/HN654-62. The molecular replacement solution was unambiguous with a correlation coefficient of ~73% after rigid body fitting. The model was refined in X-PLOR. During refinement, the peptide was omitted to reduce the potential for model bias. Density modification was performed with DM using the X-PLOR output coordinates to generate unbiased averaged electron density maps of the peptide and to fit the structure of A2.1. Unlike all of the pMHC structures that we have determined to date, the entire length of the main chain of the peptide was not visible in the electron density maps at this stage. After 10 cycles of model building and computational refinement with X-PLOR and Refmac and finally with CNS, the refinement converged. In general, the maps are clear and unambiguous. The entire A2.1 molecule is well resolved and fits the density well as evidenced by an average real space correlation coefficient of 83.8%. The positions of the termini of the HN654-662 peptide are also unambiguous and never altered through the course of refinement. However, unlike all reported pMHC structures, the center of the peptide never became clear in the density. In addition, standard 2Fo-Fc maps, simulated annealing omit maps, unaveraged omit maps and composite omit maps failed to show density for the center of the peptide. In particular, the orientation of residue 6 (Val) is completely uninterpretable and the orientation of residues 5 and 7 (Val and Gly respectively) are not well defined. This suggests that the center of the peptide is highly flexible and it does not assume one unique conformation in the center. Crystallographic data and refinement statistics are shown in Table 1. This work was completed by members of the Collins lab before I arrived.

**1.2 Using the crystallographic structure and peptide libraries, create improved HER-2/neu epitopes**

**Rationale:** The main chain of HN654-662 when bound to HLA-A2.1 will have a different path in the peptide binding cleft and the side chains will be pointing in unique orientations. This has been seen for all peptide/class I MHC molecule co-crystal structures to date. Therefore, the crystallographic structure will provide a starting point from which to improve peptide binding. The choice of which amino acid to change will depend on the orientations of the HN654-662 amino acids in the peptide binding cleft. The goal is to increase affinity without losing immunogenicity. Thus, we chose to change a residue that points down into the peptide cleft rather than up towards the TCR.

**Methods:** The crystallographic structure will tell us only which amino acid to target for changes, the best residue with which to replace the wild type sequence requires experimentation. To determine this, we will chemically synthesize small libraries of peptides that have as the root the wild type HN654-662 sequence. One amino acid in the sequence will be randomized at a time during the chemical synthesis of the peptide by substituting the wild type FMOC derivatized amino acid with a mixture of all 20 FMOC derivatized amino acids. Thus the synthesis will contain 20 different peptides (the wild type sequence serves as an internal control). This mixture will be used in our standard in vitro folding assays and folded complexes isolated. Complexes that form will be acid treated to denature the complexes and the peptide removed by filtration. The eluted peptides will be separated on a reversed-phase HPLC column and fractions analyzed by time of flight mass spectrometry to identify the peptides in collaboration with Dr. David Klapper in the department of Microbiology and Immunology. To increase the stringency, the complexes will be incubated at 37 °C to remove poorly binding peptides and separated by HPLC gel filtration before acid elution. This type of experiment will allow us to determine both the types of amino acids that may bind, and those that preferentially stabilize the complex. We expect a small number of residues to stabilize the complex based on previous work and perhaps fewer to stabilize better than the wild type sequence. Peptides that contain residues that are shown to increase stabilization will be synthesized and assayed.

**Work done:** The first library was constructed with 19 amino acids at position 3 of GP2. Cysteine was not included because of its interactions with the redox partners of glutatione in the folding buffer. Additionally, the first residue was altered to lysine to improve the solubility of the peptide library because the serine that is at position 3 in the wild-type peptide is the only polar residue in GP2 (IISAVVGIL). The initial folding experiments showed that the folded complexes were very unstable. When the purified protein was left at room temperature for a short time, all of the protein precipitated. The next preparation of recombinant protein was treated as described above (except kept at 4 °C) and analyzed by mass spectrometry by Dr. Roman Chicz, Zycos Inc. His instrument is reported to be sensitive to fmol levels of peptide. Unexpectedly, all 19 peptides were found in the spectrum of masses present from the purified protein. As the technique is extremely sensitive to the presence of the peptide, but insensitive to the amounts, it is probably not suitable for our use. We intend to repeat the experiment and perform Edman degradation on the resulting material to determine the relative amounts of each peptide present. Until we can resolve these issues, further libraries will not be made.

**1.3 Determine on-rates, off-rates and thermostability of improved epitopes**

**Rationale:** Altered sequences will demonstrate differences in physical characteristics of peptide binding. It has been shown that some peptides with decreased off-rates show greater

immunogenicity. Some HN654-662 variants that show increased binding to HLA-A2.1 will likely show greater immunogenicity.

**Methods:** These substituted peptides will be tested with the peptide transport deficient cell line T2 to determine which peptides form class I/peptide complexes with enhanced cell surface stability. We will use the cell surface stabilization assay using endogenous HLA-A2.1 in T2 cells and the conformationally specific antibody BB7.2 to asses cell surface peptide binding. Briefly cells will be incubated with peptide, washed and stained, and the mean channel fluorescence (MCF) determined by flow cytometry. To estimate off rates from stability assays, cells will be preincubated with peptides at 37 °C and Brefeldin A added to block the egress of new molecules. Aliquots of cells will be removed overtime, stained and the MCF determined as described above.

We will also measure the on and off rates of class I peptide complexes with better precision using surface plasmon resonance (Biacore). We will confirm our initial results based on the cell surface stability with soluble HLA-A2.1 and peptide using standard Biacore protocols. In brief, we will synthesize peptides with cysteine replacements at residues that are pointing towards the TCR as suggested by the crystal structure and through these cysteines the peptides will be coupled to the plasmon resonance chip. Each variant peptide will first be tested for binding to soluble HLA-A2. When the best coupling residue is identified, we will directly measure the binding compared to both the wild type epitope and non-binding controls. To determine the magnitude of the difference in peptide affinity as a result of changing an amino acid to cysteine, we will perform competition binding experiments using an unrelated HLA-A2.1 binding peptide on the chip to compete for binding with both the wild type and altered peptides. Alternatively we can perform the entire assay as a competitive binding assay, using the Biacore signal read out. We do wish to note that the measurement of stability on cells at 37 °C may better reflect *in vivo* stability than the Biacore measurement, while the Biocore will allow much greater precision and accuracy of the measurements

Measurement of the overall thermostability of the peptide/class I complex by circular dichroism (CD) spectroscopy provides a measure of the overall stability of the complex. The thermodynamic stability will be measured using the extracellular portion of HLA-A2.1 produced in E. coli and folded *in vitro* in the presence of the appropriate synthetic peptide. The melting temperature, an indication of the stability of the complex, will be measured by following the loss of circular dichroic signal as a function of temperature. The thermodynamic data will be correlated with the observed half-lives of these peptides bound to the MHC class I molecule for a full description of the binding of each peptide. While a melting temperature in excess of 37 °C may not seem physiologically important, they do reflect the thermodynamic stability of the complexes and will likely yield data important for creating the best binding peptide if a third generation is required.

**Work done**: We have chemically synthesized the peptides shown in Table 1 and determined their on-rates, off-rates and melting temperatures as described previously. The melting temperature for HN654-662 is 36.4 °C. This complex has the lowest $T_m$ of any complex measured to date. This peptide is perfect example for poor-binding peptide and offers the first opportunity to understand poor binding. Substitutions at peptide anchor positions (I/2L, L9V and I2L/L9V) and at both anchor and middle of the peptide (I2L/V5L, I2L/V5L/L9V, V5L/L9V) shown in Table II did not improved the thermal stability significantly (~2-6 °C). Similar substitutions increased the stability drastically in the influenza matrix peptide (~7-9 °C) and for a variant of melanoma peptide (MelA and MelA-A2L (~9 °C). Only V5L is more

stable with higher melting temperature ($T_m$ = 45.8 °C). A cell surface binding assay using T2 cells with exogenously added peptide confirms the results found by the circular dichroism studies. Two peptides, one hydrophobic and one hydrophilic, were chosen as representative "high affinity" binders. ML is derived from the signal sequence of calreticulin and RT is derived from HIV-1 reverse transcriptase. The thermal stability ($T_m$) and relative binding constant ($K_r$) determined by T2 assay correlate well (91.3% correlation coefficient).

Adding brefeldin A (BFA) to the cell surface stability assay allows us to measure the amount of time a peptide/MHC complex stays on the surface of the cells. The HN654-662 peptide has an extremely short half life ~21 minutes at 37 °C. The half lives of altered peptides/MHC complexes on the cell surface are increased with respect to HN654-662 peptide. However, they are not close to the time constants seen for the positive control, high affinity binders ML or RT.

We plan to measure the on and off rates of class I peptide complexes with better precision using surface plasmon resonance (Biacore). Based on these data above and the crystallographic structure, we have determined that we can replace the isoleucine at position 8 with a cysteine in order to perform these experiments. However, our recent data describing interactions between the pockets and other residues in the peptide (described below) is potentially more exciting and this particular experiment is on hold.

**1.4 Determine on rates, off rates and thermostability of improved epitopes in the presence of physiological osmolytes**

**Rationale:** MHC complexes *in vivo* exist in a concentrated environment. Osmolytes have been shown to stabilize protein structures and are present in the cellular environment. Physiological concentrations of osmolytes have been shown to preferentially stabilize the poor affinity peptide HN654-662 bound to HLA-A2.1. The affinity and stability of substituted peptides and complexes should be analyzed in the presence of physiological concentrations of osmolytes for a better estimate of overall stability. Osmolytes have not been in previous stability studies of HLA complexes, so we will conduct both sets of experiments to get a better understanding of the degree of stabilization achieved. In addition this information might lead to better crystallization conditions for the parent and substituted peptides; this would facilitate the final structures.

**Methods:** We will repeat the procedures described in the methods of section 1.3. Briefly, substituted peptides will be tested in the cell surface stabilization assay using endogenous HLA-A2. 1 in T2 cell and the conformationally specific antibody BB7.2 to assess cell surface peptide binding as described before. Cells will be incubated with peptide in serum-free media with different osmolytes concentrations, washed and stained, and the mean channel fluorescence (MCF) determined by flow cytometry. Biacore measurements and CD measurements will also be performed in the presence of different concentrations of osmolytes centered around the physiological value. In addition to sodium chloride, glucose and sucrose will be examined. Other physiological osmolytes will be tested as time permits.

**Work done:** We have focused on the mechanism of NaCl thermal stabilization. As described, the thermal stability of these complexes are maximal in the presence of 150 mM NaCl. The potential mechanisms for stabilization are changes in water interactions with the protein by inclusion of NaCl or by direct binding of the salt. Since HLA-A2.1 is actually a ternary complex, the effect could be due to physical stabilization any one of the pieces: peptide, heavy chain or β2m or by changing the interactions of any of the pieces. We have shown that β2m is

not stabilized in the presence of increasing concentrations of salt (heavy chain is not soluble in the absence of peptide or β2m). However, we have seen increases in thermal stability when β2m is covalently linked to the MHC complex. This leads us to believe that the mechanism of thermal stabilization is the increased affinity of β2m for heavy chain. This protein acts as a wedge keeping the peptide-binding superdomain closed so that peptide cannot escape.

**1.5 Determine the mechanism for the loss of thermal stability of I2L/V5L compared to I2L and V5L alone.**

**Rational:** The thermal stability of HN654-662 variants is higher when there is only single substitution i.e. either in middle or at anchor position. There was a loss of thermal stability in the variants where substitutions were at both middle and anchor positions. Understanding the reasons for loss of thermal stability in variants having both substitutions as compared to the variants with single substitutions would help to understand the mechanism of binding and allow us to probe how residues influence each other in the peptide

**Method:** The crystallographic structures of complexes of HLA-A2.1 and variants with single substitution and with two or more substitutions at both anchor position and in the middle of the peptide will help in understanding the mechanism of binding. The resulting structures will be compared with the crystal structure of HLA-A2.1 plus wild type and among themselves.

**Work done:** In order to understand why substitutions at anchor positions together with substitutions at the middle of the peptide results in loss of thermal stability as compared to single substitutions either at anchor position or in the middle, the crystal structures of HLA-A2.1/HN654 variants (I2L/V5L/L9V) and (I2L/V5L). The molecular replacement solution gave a correlation coefficient of ~ 79% for I2L/V5L/L9V and ~75% for I2L/V5L. The models were refined in CNS and peptide was omitted during refinement to reduce the bias. Density modification was performed with DM to generate unbiased averaged electron density maps of the peptide. The model building was performed using O. The electron density at the center of the peptide is not well defined in both structures. This is quite similar to the structure of wild type. This shows that the center of the peptide is highly flexible. In V5L, having higher melting temperature ($T_m$ = 45.8 °C), we expect that substituted Leu would point in the binding cleft and make stabilizing contacts. Surprisingly the double or triple variants are less stable than single substitutions. The crystal structures of I2L/V5L/L9V ($T_m$ = 39.5 °C) shows that substituted Leu at fifth position point away from the MHC molecule while that of I2L/V5L ($T_m$ = 39.0 °C) shows that the orientation of substituted Leu at fifth position is same as that of Val 5 in wild type. It seems that substitutin of either of the anchor residues makes the position 5 Leu point away from class I MHC. We are trying to crystallize and solve the crystal structure of V5L and I2L variants. The structure of V5L variant will help in understanding the stabilizing contacts for strong binding.

## Appendix

**Crystallographic structure of A2/HN654-662 variant (I2L/V5L/L9V).** In order to understand why substitutions at anchor positions together with substitution at middle of the peptide do not significantly improve the binding affinity, the crystal structure of A2/HN654-662 variant (I2L/V5L/L9v) was determined. The molecular replacement solution gave a correlation coefficient of .~ 79% after rigid body fitting. The model was refined in CNS and during refinement the peptide was omitted to reduce the potential bias. Density modification was performed with DM to generate unbiased avearged electron density map of the peptide and to fit the structure of A2. The model building was performed using O. The crystallographic data and refinement statistics are shown in Table 3. We expected substituted Leu at fifth position to point down ward in the binding cleft and make stabilizing contact with A2 molecule but it points away from the MHC molecule. It seems that the substitution of the anchor residues makes the substituted Leu at fifth position to point away from the peptide binding cleft. In V5L, which has higher melting temperature (Tm = 45.8 C), we expect that substituted Leu would point in the binding cleft and make stabilizing contacts.

## Key Research Accomplishments

- The X-ray crystal structure of the complex of human HLA-A2 plus HN654-662 peptide (IISAVVGIL) was determined to 2.4 Å resolution. The structure suggests that the center of the peptide is highly flexible which may account for its poor binding.

- Thermal denaturation studies of various complexes of HLA-A2 with HN654-662 or an altered peptide ligand provided a measure of overall complex stability. HLA-A2/HN654-662 complex is very unstable (Tm=36.6°C). We have made many variants, only one, V5L, is relatively more stable with higher Tm (Tm =45.8°C).

- The X-ray crystal structure of HLA-A2 plus HN654-662 variant (I2L/V5L/L9V) was determined to 2.25Å resolution. This MHC-I/peptide complex is slightly more stable (Tm=39.5°C) than its wild type but less stable than V5L. We expect the substituted Leu to point downwards and interact strongly with the MHC molecule but it seems that the substitution of the anchor residues in I2l/V5L/L9V makes the Leu at the fifth position point away from the MHC molecule.

## Manuscripts

Jennifer J. Kuhns, Michael A. Batalia, Shuquin Yan and Edward J. Collins (1999) Poor binding of a HER-2/neu Epitope (GP2) to HLA-A2.1 is due to a lack of interactions with the center of the peptide. *J. Biol. Chem.* 274, 36422-36427.

A2/IISAVVGIL (GP2)

| Parameters | | Refinement | |
|---|---|---|---|
| **Space group P1** | **Resolution** | | **30 - 2.4 Å** |
| Cell Dimensions | a = 50.34 Å | $R_{work}$[3] (31,969) | 24.2% |
| | b = 63.61 Å | $R_{free}$ (1714) | 28.4% |
| | c = 75.14 Å | Error[5] | 0.26Å |
| | α = 81.98° | Non-hydrogen atoms | 6292 |
| | β = 76.25° | <RS fit>[4] | 83.8% |
| | γ = 77.83° | Average B factor | 16.8Å$^2$ |
| | | No. Of waters | 103 |
| Molecules/ AU | 2 | RMSD | |
| Resolution | 2.4 Å | bonds | 0.009Å |
| Number of Crystals | 1 | angles | 1.468° |
| $R_{merge}$(%)[1] | 9.3 (23.3) | | |
| <I/σ> | 7.8 (3.46) | Ramachandran | |
| Unique reflections | 34,962 | Most favorable | 91.6% |
| Total observations | 66,839 | Additionally allowed | 8.1%% |
| Completeness (%) | 98.2 (97.6) | Generously allowed | 0.3% |
| | | Disallowed | 0.0% |

Table I. Summary of crystallographic and refinement statistics.

1. $R_{merge} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$, where $I_i$ is the observed intensity and <I> is the average intensity of multiple observations of symmetry related reflections.

2. Number in parenthesis refers to the highest resolution shell (2.44 – 2.40Å).

3. $R = \sum_{hkl} | |F_{obs}| - k |F_{cal}| | / \sum_{hkl} |F_{obs}|$, where $R_{free}$ is calculated for a randomly chosen 5% of reflections, $R_{work}$ is calculated for the remaining 95% of reflections used for structure refinement. Numbers in parenthesis refer to the number of structure factors used in the measurements.

4. Rs fit is the average real space fit of all atoms on an electron density map from DM with two fold non crystallographic averaging, histogram matching and solvent flattening

5. Error is the mean coordinate error estimate based on maximum likelihood measurements (27).

| Peptide | Sequence | $T_m$ | $K_r$ | $T_{1/2}$ |
|---|---|---|---|---|
| HN654-662 | IISAVVGIL | 36.4 | >50 | 0.35 |
| I2L | **L**LSAVVGIL | 42.2 | 22.9 | 1.76 |
| L9V | IISAVVGI**V** | 38.8 | >50 | 0.69 |
| I2L/L9V | **L**LSAVVGI**V** | 42.5 | 10 | 2.48 |
| V5L | IISA**L**VGIL | 45.8 | | |
| I2L/V5L | **L**LSA**L**VGIL | 39 | 49.3 | 1.059 |
| V5L/L9V | IISA**L**VGI**V** | 38.8 | >50 | 1.1769 |
| I2L/V5L/L9V | **L**LSA**L**VGI**V** | 39.5 | 17.5 | 1.075 |
| ML | MLLSVPLLL | 52.5 | 1.8 | 19.53 |
| RT | ILKEPVHGV | 50.0 | 7.7 | 9.69 |
| MelA | EAAGIGILTV | 40.9 | 47.2 | 0.44 |
| MelA-A2L | ELAGIGILTV | 50.0 | 1.6 | 9.98 |

**Table II. Summary of Binding data of HER-2/neu derived peptides to A2.** Residues substituted with respect to wild type peptide are shown in boldface. $T_m$ is the temperature (°C) at which 50% of protein is denatured as measured by circular dichroism. $K_r$ is the relative binding constant as determined by the T2 cell surface assembly assay. $K_r$ is defined as concentration of peptide in uM that yields 50% mean channel fluorescence (MCF) as compared to the maximum fluorescence of the control peptide (ML) at 50 uM. The $K_r$ value for ML is the concentration that yields MCF. $T_{1/2}$ is the half life of peptide/A2 complexes (in hours) as determined by the T2 cell surface stability assay. The error in the $T_m$ is the sum of machine and curve fit errors. It is typically about 1 °C. >50 means that the concentration to yield 50% of ML fluorescence is greater than 50 uM.

A2/ILSALVGIV

| Parameters | | Refinement | |
|---|---|---|---|
| **Space group P1** | **Resolution** | **50 - 2.25 Å** | |
| Cell Dimensions | a = 49.95 Å | $R_{work}$[3] (36,189) | 24.8% |
| | b = 62.93 Å | $R_{free}$ (1907) | 28.6% |
| | c = 74.65 Å | Error[5] | 0.26Å |
| | α = 82.07° | Non-hydrogen atoms | 6292 |
| | β = 76.50° | <RS fit>[4] | 84.0% |
| | γ = 78.04° | Average B factor | 19.1Å$^2$ |
| | | No. Of waters | 144 |
| Molecules/ AU | 2 | RMSD | |
| Resolution | 2.25 Å | bonds | 0.007Å |
| Number of Crystals | 1 | angles | 1.3° |
| $R_{merge}$(%)[1] | 5.5 (18.0) | dihedrals | 24.9° |
| <I/σ> | 14.0 (5.7) | Ramachandran | |
| Unique reflections | 38,096 | Most favorable | 91.6% |
| Total observations | 214687 | Additionally allowed | 8.1%% |
| Completeness (%) | 93.8(72.0) | Generously allowed | 0.3% |
| | | Disallowed | 0.0% |

Table III. Summary of crystallographic and refinement statistics.

1. $R_{merge} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$, where $I_i$ is the observed intensity and <I> is the average intensity of multiple observations of symmetry related reflections.

2. Number in parenthesis refers to the highest resolution shell (2.44 – 2.40Å).

3. $R = \sum_{hkl} | |F_{obs}| - k |F_{cal}| | / \sum_{hkl} |F_{obs}|$, where $R_{free}$ is calculated for a randomly chosen 5% of reflections, $R_{work}$ is calculated for the remaining 95% of reflections used for structure refinement. Numbers in parenthesis refer to the number of structure factors used in the measurements.

4. Rs fit is the average real space fit of all atoms on an electron density map from DM with two fold non crystallographic averaging, histogram matching and solvent flattening

5. Error is the mean coordinate error estimate based on maximum likelihood measurements (27).

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# Poor Binding of a HER-2/neu Epitope (GP2) to HLA-A2.1 Is due to a Lack of Interactions with the Center of the Peptide*

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**Class I major histocompatibility complex (MHC) molecules bind short peptides derived from proteins synthesized within the cell. These complexes of peptide and class I MHC (pMHC) are transported from the endoplasmic reticulum to the cell surface. If a clonotypic T cell receptor expressed on a circulating T cell binds to the pMHC complex, the cell presenting the pMHC is killed. In this manner, some tumor cells expressing aberrant proteins are recognized and removed by the immune system. However, not all tumors are recognized efficiently. One reason hypothesized for poor T cell recognition of tumor-associated peptides is poor binding of those peptides to class I MHC molecules. Many peptides, derived from the proto-oncogene HER-2/neu have been shown to be recognized by cytotoxic T cells derived from HLA-A2$^+$ patients with breast cancer and other adenocarcinomas. Seven of these peptides were found to bind with intermediate to poor affinity. In particular, GP2 (HER-2/neu residues 654–662) binds very poorly even though it is predicted to bind well based upon the presence of the correct HLA-A2.1 peptide-binding motif. Altering the anchor residues to those most favored by HLA-A2.1 did not significantly improve binding affinity. The crystallographic structure shows that unlike other class I-peptide structures, the center of the peptide does not assume one specific conformation and does not make stabilizing contacts with the peptide-binding cleft.**

Class I major histocompatibility complex (MHC)[1] proteins bind short peptides (9–11 amino acids) derived from cytosolically degraded proteins. These peptides are transported into the endoplasmic reticulum and bind to newly formed class I molecules. Peptide binding appears to be the final step in assembly of the complex (1). Following peptide binding, the complexes are transported to the plasma membrane. At the plasma membrane, clonotypic T cell receptors on the surface of circulating cytotoxic T lymphocytes (CTL) may recognize the peptide-MHC complex (pMHC). If the pMHC is recognized by the T cell receptor, the T cell is activated and the cell presenting the pMHC is killed. A normal cell will have a large assortment of pMHC on the cell surface that are not recognized by CTL. However, viral or mutated self-proteins are degraded by these same mechanisms, and many of the resulting pMHC are recognized by CTL. In this manner, virus-infected or mutated cells are targeted for lysis by cytotoxic T cells (reviewed in Ref. 2). Self-proteins that are expressed in abnormally high amounts or in abnormal cell types may also be targets for CTL (3).

Class I MHC molecules bind many peptides with diverse sequences and high affinity (4). To bind all these peptides, the class I protein primarily interacts with the invariant portions of the peptides, the N and C termini (5). Class I MHC also uses a subset of amino acid side chains within the peptide termed "anchors" to generate significant binding (6). These peptide anchor residues bind within "specificity pockets" that are primarily formed by the polymorphic residues within the peptide-binding cleft of the MHC molecule (7). Peptides that bind with high affinity to a given allotype are typically found to have one of a few preferred amino acids at each anchor position. The corresponding hypothesis is that peptides that do not have those preferred amino acids at the anchor positions will not bind well. The combination of amino acids that may bind at the anchor positions is known as the peptide-binding motif (8). These motifs have proven to be extremely valuable in predicting peptides that will bind to class I MHC. Other residues within the peptide besides the anchors may be used to generate increased binding affinity (9–11).

Interestingly, many peptides that appear to have the correct peptide-binding motif still bind poorly. Substituting the anchor residues of poor binding peptides with those that are most preferred by the allotype can generate high affinity binding. (10, 12). Some of these altered peptide ligands (APL) are even effective therapeutics (13). We show here that there are also peptides for which altering the anchor residues does not significantly increase binding affinity. It is not clear from the previously available data in the literature why these peptides bind poorly.

HER-2/neu (c-erb-2) encodes a receptor tyrosine kinase with homology to the epidermal growth factor receptor. Overexpression of HER-2/neu in many adenocarcinomas, including breast and ovarian tumors, correlates with a poor prognosis for remission and recovery (14). Tumor infiltrating lymphocytes have been found in cancer patients that overexpress HER-2/neu, and these tumor infiltrating lymphocytes are able to recognize and lyse the solid tumor (3, 15, 16), but these CTL do not eliminate the tumor. It has also been shown that several peptide epitopes derived from the gene product of HER-2/neu are presented by class I MHC molecules to circulating CTL. As with many other

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*The atomic coordinates and structure factors (code 1QR1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).*

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[1] The abbreviations used are: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; pMHC, peptide-MHC complex(es); APL, altered peptide ligand(s); HPLC, high pressure liquid chromatography; BFA, brefeldin A; A2, HLA-A2.1.

tumor-associated antigens, most of these peptides bind poorly to HLA-A2.1 (A2). There are many potential reasons for the lack of immune removal of tumors including the down-regulation of class I MHC or down-regulation of the protein from which the peptide is derived. It has also been proposed that one reason for poor recognition by CTL is weak binding of the immunogenic peptides to class I MHC (3).

Here we show that HER-2/neu-derived peptides, identified in the literature as recognized by CTL, bind with a range of affinities, but all are lower affinity than two index peptides of high affinity. One peptide was chosen for further study. This peptide, GP2 (IISAVVGIL), binds very poorly to A2 but has anchor residues that are present in high affinity peptides (Ile at position 2 and Leu at position 9). Its inherently poor affinity is not significantly increased by substitution of its anchor residues. To understand why this peptide binds poorly, the crystallographic structure of the A2-GP2 complex was determined. Unlike all previously determined peptide-class I MHC (pMHC) structures, there is a large region of unresolved electron density in the center of the peptide. We interpret this to mean that the peptide assumes more than one conformation within the peptide-binding cleft. We hypothesize that the observed poor binding is due to the lack of important secondary interactions within the center of the peptide.

## EXPERIMENTAL PROCEDURES

*Preparation of HLA-A2.1-Peptide Complexes*—Residues 1–275 of HLA-A2.1 (A2) and residues 1–99 of human $\beta_2$-microglobulin were expressed in *Escherichia coli*, produced as inclusion bodies, purified, and folded as described previously (17). Briefly, peptide, solubilized $\beta_2$-microglobulin and solubilized A2 heavy chain were rapidly diluted into folding buffer (10 mM Tris, pH 8.0, 0.4 M L-Arg, 10 mM reduced glutathione, 1 mM oxidized glutathione, and protease inhibitors) at molar ratios of 10:5:1, respectively. The final protein concentration was kept below 50 $\mu$g ml$^{-1}$. The solution was incubated at 10 °C for 36–48 h and then concentrated (Amicon) and purified by HPLC gel filtration (Phenomenex, BioSep-SEC-S2000).

*Synthetic Peptides*—All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina at Chapel Hill. The peptides were purified to greater than 95% purity by reversed-phase HPLC and identity confirmed by matrix-assisted laser desorption ionization-time-of-flight spectroscopy. Peptides were dissolved in 100% dimethyl sulfoxide at 20 mg ml$^{-1}$ by weight. The final peptide concentration was determined by amino acid analysis (Protein Chemistry Laboratory, Department of Chemistry, University of North Carolina, Chapel Hill). The list of peptides and references for immunogenicity are given in Table I.

*Determination of Thermal Stability*—Purified A2-peptide complexes were exchanged into a 10 mM $KH_2/K_2HPO_4$ buffer, pH 7.5, and adjusted to a final protein concentration of 4–12 $\mu$M. The change in CD signal at 218 nm was measured as a function of temperature from 4 to 95 °C on an AVIV 62-DS spectropolarimeter (Aviv Associates Inc, Lakewood, NJ). The final melting curve was the average of at least three experiments for each A2-peptide complex. $T_m$ values were calculated as the temperature at which 50% of the complexes are denatured using a two-state denaturation model (12).

*Cell Surface Stabilization Assay*—Cell surface stabilization of A2 was performed as described previously (11). Briefly, $2.5 \times 10^5$ T2 cells (ATCC CRL-1992) were incubated overnight in AIM V serum-free medium (Life Technologies, Inc.) at 37 °C, 5% $CO_2$ in the presence of GP2 or APL, at concentrations varying from 50 to 0.05 $\mu$M. Cells were then stained with the monoclonal antibody BB7.2 (18) specific for A2, followed by fluorescein isothiocyanate-labeled (1:50) goat anti-mouse IgG antibody (Southern Biotechnology Associates). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson), and the mean channel fluorescence was determined using the CYCLOPS software package (Cytomation, Fort Collins, CO). All data are normalized as the percentage of the mean channel fluorescence for the calreticulin-signal-sequence peptide, ML, bound to A2 at 50 $\mu$M. Binding by the A2-specific antibody, BB7.2, was not dependent on the peptide bound because W6/32, an antibody that binds to an epitope between the $\alpha$3 domain and $\beta_2$-microglobulin, gave similar results (data not shown).

*Cell Surface Half-life Assay*—The determination of cell surface half-lives ($T_{1/2}$) of A2-peptide complexes was performed as described previously (11). Briefly, $2.5 \times 10^6$ T2 cells were incubated overnight in AIM V serum-free medium at 37 °C, 5% $CO_2$ in the presence of 50 $\mu$M peptide. To block the egress of new A2 molecules to the surface, cells were incubated at 37 °C, 5% $CO_2$ in RPMI 1640, 15% fetal calf serum and 10 $\mu$g ml$^{-1}$ brefeldin A (BFA, Sigma). This concentration of BFA is toxic to the cells. Therefore, after 1 h the cells were then transferred to RPMI 1640, 15% fetal calf serum, and 0.5 $\mu$g ml$^{-1}$ BFA. At the indicated time points, $2.5 \times 10^5$ cells were removed, incubated with BB7.2, and analyzed by flow cytometry as described above for cell surface stabilization assay. Each time point is evaluated as mean fluorescence with peptide minus mean fluorescence without peptide and normalized to the maximal level of fluorescence (at time zero) for each peptide.

TABLE I

*Summary of binding data of HER-2/neu-derived peptides to A2*

Residues substituted with respect to wild-type peptide are shown in boldface type. $T_m$ is the temperature (°C) at which 50% of the protein is denatured as measured by circular dichroism. $K_r$ is the relative binding constant as determined by the T2 cell surface assembly assay. $K_r$ is defined as the concentration of peptide in $\mu$M that yields 50% mean channel fluorescence as compared to the maximum fluorescence of the control peptide (ML) at 50 $\mu$M. The $K_r$ value for ML is the concentration that yields 50% mean channel fluorescence. $T_{1/2}$ is the half-life of peptide-A2 complexes (in hours) as determined by the T2 cell surface stability assay. ND, not determined. DNF, did not fold *in vitro*. The error in the $T_m$ is the sum of the machine and curve fit errors. It is typically about 1 °C. >50 means that the concentration to yield 50% of ML fluorescence is greater than 50 $\mu$M.

| Peptide | Sequence | $T_m$ | $K_r$ | $T_{1/2}$ |
|---|---|---|---|---|
| GP2 (16) | IISAVVGIL | 36.4 | >50 | 0.35 |
| S1 (38) | SIISAVVGI | 44.2 | 13.2 | 4.05 |
| L10 (38) | IISAVVGILL | 41.3 | >50 | 1.58 |
| E74 (15) | DVRLVHRDL | DNF | >50 | DNB |
| E75 (15) | KIFGSLAFL | 45.1 | 14.4 | 8.57 |
| F56 (39) | YISAWPDSL | 34.8 | >50 | DNB |
| C84 (39) | ELVSEFSRV | 37.5 | >50 | 0.30 |
| L9V | IISAVVGI**V** | 38.8 | >50 | 0.69 |
| I2L | I**L**SAVVGIL | 42.2 | 22.9 | 1.76 |
| I2L/L9V | I**L**SAVVGI**V** | 42.5 | 10.0 | 2.48 |
| ML (30) | MLLSVPLLL | 52.5 | 1.8 | 19.53 |
| RT (31) | ILKEPVHGV | 50.0 | 7.7 | 9.69 |
| MelA (36) | EAAGIGILTV | 40.9 | 47.2 | 0.44 |
| MelA-A2L | E**L**AGIGILTV | 50.0 | 1.6 | 9.98 |

*Crystallization, Data Collection and Processing*—Crystals were grown by hanging drop vapor diffusion as described previously (19). Crystallographic data of A2-GP2 were collected on a single crystal at the National Synchrotron Light Source, Brookhaven National Labs, beamline X-12B at −170 °C (Oxford Cryosystems). Evaluation of the diffraction pattern with DENZO autoindexing function showed the space group to be triclinic P1. 180 degrees of data were collected at a distance of 90 mm from the Quantum 4 CCD detector (ADSC Poway, CA) with one-degree oscillations and 3 min of exposure time/frame. Data were processed with DENZO, and intensities were scaled with SCALEPACK (20). Data statistics are shown in Table II.

*Structure Determination and Refinement*—The A2-GP2 structure was determined by molecular replacement using AMoRe (21) within the CCP4 program suite (22). The A2-hepatitis peptide complex (PDB accession code 1HHH) was used as the search model (23). Because the domains tend to vary in their relative orientations with respect to one another in different crystal forms, the search model was divided into three pieces, the peptide-binding superdomain ($\alpha_1\alpha_2$), the $\alpha_3$ domain, and $\beta_2$-microglobulin. Initial rounds of positional refinement used X-PLOR from 8–2.4 Å resolution data. Later rounds were performed with Refmac using all data from 30.0 to 2.4 Å. Final rounds of refinement used torsional dynamics with CNS (24–26) with all data. Electron density maps were generated using DM and functions for 2-fold non-crystallographic averaging, histogram matching, and solvent flattening. Manual intervention was performed using O (27). 103 water molecules were added to the structure using the program ARP (28) combined with Refmac and confirmed by visual inspection of the electron density maps. The refinement statistics are listed in Table II.

## RESULTS

*HER-2/neu-derived Peptides Bind Poorly to A2*—We began these studies to assess the correlation of immunological activity with peptide binding affinity to HLA-A2.1 (A2). Thermal stability of class I MHC-peptide complexes, as measured by

TABLE II
*Summary of crystallographic data*

The crystallographic structure of A2-GP2 was determined by molecular replacement using the A2-hepatitis B 10-mer (Protein Data Bank code 1HHH) as the search model. The structure was refined by a combination of X-PLOR and Refmac. Individual Bs were refined in the penultimate cycle followed by the addition of waters.

| | |
|---|---|
| **Data statistics** | |
| Space group | P1 |
| Cell Dimensions | $a$ = 50.34 Å<br>$b$ = 63.61 Å<br>$c$ = 75.14 Å<br>$\alpha$ = 81.98 °<br>$\beta$ = 76.25 °<br>$\gamma$ = 77.83 ° |
| Molecules/Asymmetric Unit | 2 |
| Resolution | 30–2.4 Å |
| $R_{merge}$ (%)[a] | 9.3 (23.3)[b] |
| $\langle I/\sigma \rangle$ | 7.80 (3.46) |
| Unique reflections | 34,962 |
| Total reflections | 66,839 |
| Completeness (%) | 98.2 (97.6) |
| **Refinement** | |
| Resolution | 30–2.4 Å |
| $R_{free}$ (%) (number of reflections)[c] | 28.4 (1,714) |
| $R_{work}$ (%) (number of reflections)[c] | 24.2 (31,969) |
| *Rs* fit[d] | 83.8% |
| No. of non-hydrogen atoms | 6,292 |
| No. of waters | 103 |
| Error[e] | 0.26 Å |
| Average B factor | 16.8 Å$^2$ |
| R.M.S. deviations from ideality | |
| Bonds | 0.009 Å |
| Angles | 1.468 ° |
| Residues in Ramachandran plot | |
| Most favored | 91.6% |
| Additional allowed | 8.1% |
| Generously allowed | 0.3% |
| Disallowed | 0.0% |

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i - \langle I \rangle| / \Sigma_{hkl} \Sigma_i I_i$, where $I_i$ is the observed intensity and $\langle I \rangle$ is the average intensity of multiple observations of symmetry related reflections.

[b] Number in parenthesis refers to the highest resolution shell (2.44–2.40) for A2-GP2 unless otherwise stated.

[c] $R = \Sigma_{hkl} ||F_{obs}| - k|F_{cal}|| / \Sigma_{hkl} |F_{obs}|$, where $R_{free}$ is calculated for a randomly chosen 5% of reflections and $R_{work}$ is calculated for the remaining 95% of reflections used for structure refinement.

[d] *Rs* fit is the average real space fit of all atoms on an electron density map from DM with 2-fold noncrystallographic averaging, histogram matching, and solvent flattening.

[e] Error is the mean estimate of the coordinate error based on maximum likelihood methods (Refmac).

circular dichroism, have been shown to correlate with the free energy of peptide binding to class I MHC (29). Therefore, the thermal stabilities of recombinant A2 complexes folded *in vitro* with seven HER-2/neu peptides identified as important epitopes for breast cancer immunotherapy in the literature (GP2, S1, L10, E74, E75, F56, and C84; see Table I for sequences) were determined. As can be seen from Fig. 1*A*, complexes formed with GP2, F56, and C84 have extremely low melting temperatures. Complexes formed with S1, L10, and E75 (summarized in Table I) have higher melting temperatures. E74 bound so poorly as to be undetectable in any of our assays (data not shown). A cell surface binding assay (Fig. 1*B*) using T2 cells with exogenously added peptide confirms the results found by the circular dichroism experiments. Two peptides, one hydrophobic and one hydrophilic, were chosen as representative "high affinity" binders. ML is derived from the signal sequence of calreticulin (30), and RT is derived from HIV-1 reverse transcriptase (31). The thermal stability ($T_m$) and the relative binding constant ($K_r$) determined by the T2 assay correlate well (91.3% correlation coefficient). This suggests that $K_r$ is proportional to $K_D$, because, as stated above, the $T_m$ has previously been shown to be proportional to the $K_D$ (29).

Adding BFA to the cell surface stability assay allows us to measure the amount of time a peptide-MHC complex stays on the surface of cells. As can be seen in Fig. 1*C*, the GP2 peptide has an extremely short half-life of ~21 min at 37 °C (Table I). Some of the other HER-2/neu-derived peptides have longer half-lives, but none are as long as peptides such as ML or RT (Fig. 1*C* and Table I). We could not detect binding of the E74 peptide in any of our assays. The fact that CTL activity toward E74 can be seen and we cannot measure binding is probably a function of the extreme sensitivity of CTL. Only ~100 class I-peptide complexes/cell are required to trigger an activated T cell (32).

*Anchor Substitutions Do Not Significantly Improve Binding of GP2*—Substitutions at peptide anchor positions have been shown to greatly increase the thermal stability of an influenza matrix peptide (12). In the present work, we chose one of the three poor binding peptides, GP2, to study the anchor substitutions of HER-2/neu-derived peptides. The GP2 anchors (Ile at position 2 and Leu at position 9 of the peptide) are found in peptides that bind with high affinity to A2, but these anchors are not optimal (8). Therefore, optimized APL based on GP2 were synthesized that replaced the Ile at position 2 with Leu (I2L) or the Leu at position 9 with Val (L9V). As can be seen in Fig. 1*D*, these substitutions did increase the thermal stability (~2–6 °C) but not to the degree that was seen for similar substitutions in the influenza matrix peptide (~7–9 °C) (12) or for a variant of a melanoma peptide (MelA and MelA-A2L, ~9 °C). The cell surface stability assay using T2 cells supports the CD data that we have measured (Fig. 1*E*). The half-lives of the APL complexes on the cell surface are increased with respect to GP2 (Fig. 1*F*). However, they are not close to the time constants seen for the positive control, high affinity binders ML or RT.

*Crystallographic Structure of A2-GP2*—To understand why GP2 binds poorly to A2 and why the anchor substitutions do not significantly increase the stability, we determined the crystallographic structure of A2-GP2. The molecular replacement solution was unambiguous with a correlation coefficient of ~73% after rigid body fitting. The model was refined in X-PLOR (33). During refinement, the peptide was omitted to reduce the potential for model bias. Density modification was performed with DM (22) using the X-PLOR output coordinates to generate unbiased averaged electron density maps of the peptide and to fit the structure of A2. Unlike all of the pMHC structures that we have determined to date, the entire length of the main chain of the peptide was not visible in the density modified electron density maps at this stage. After 10 cycles of model building with O (27) and computational refinement with X-PLOR and Refmac and finally with CNS, the refinement converged to the statistics shown in Table II. In general, the maps are clear and unambiguous. The entire A2 molecule is well resolved and fits the density well as evidenced by an average real space correlation coefficient of 83.8%. The positions of the termini of the GP2 peptide are also unambiguous and never altered through the course of refinement. However, unlike all reported pMHC structures, the center of the peptide never became clear in the density (Fig. 2). In addition, standard $2F_o - F_c$ maps, simulated annealing omit maps, unaveraged omit maps, and composite omit maps failed to show density for the center of the peptide. In particular, the orientation of residue 6 (Val) is completely uninterpretable, and the positions of residues 5 and 7 (Val and Gly, respectively) are not well defined.

## DISCUSSION

One hypothesis used to explain why tumors are not recognized and eliminated by the immune system is that potentially

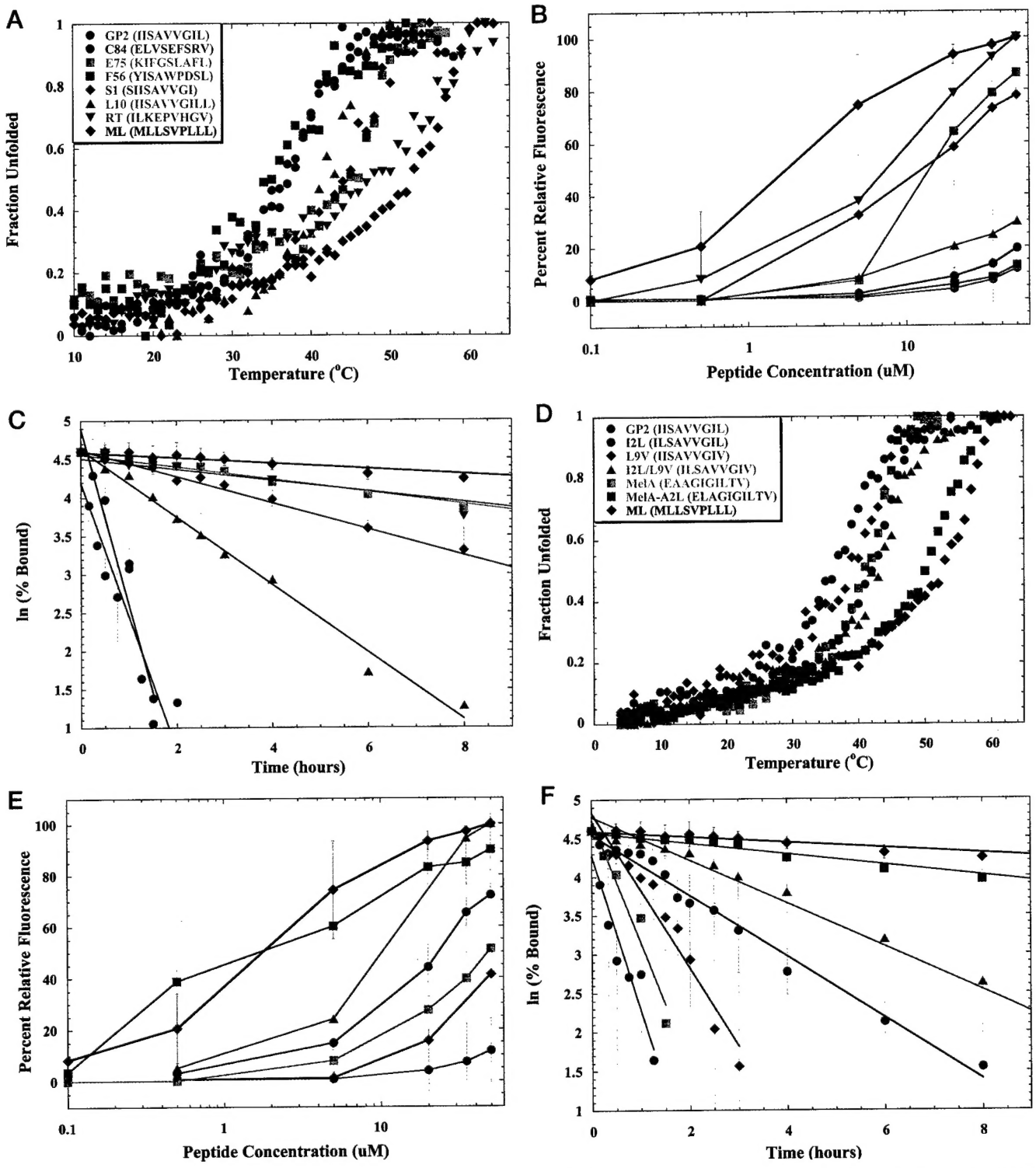


FIG. 1. **HER-2/neu-derived peptides bind poorly to A2, and anchor substitutions do not increase the stability of GP2-derived APL.** The symbols and colors shown in *A* are also those used in *B* and *C*. Likewise, the symbols and colors shown in *D* are also those used in *E* and *F*. *A*, thermal stability of A2-peptide complexes as measured by CD. 4–12 $\mu$M protein was denatured by heat in a circular dichroism spectropolarimeter. The change in CD signal at 218 nm is an indication of the loss of secondary structure within the protein. Each curve is the average of three independent experiments. The error in the $T_m$ is the sum of the curve fit error and the Peltier temperature controller error and is ~1 °C. *B*, cell surface measurements confirm relative affinities measured by circular dichroism. T2 cells were incubated with the indicated concentrations of peptide and the amount of cell surface A2 measured by flow cytometry using an A2-specific monoclonal antibody BB7.2. *C*, cell surface half-lives of A2-peptide complexes were determined by treating the peptide-pulsed cells (as in *B*) with BFA to halt vesicular transport. Aliquots of cells were removed at the indicated times and the remaining A2 on the cells determined by incubating with BB7.2. *D*, CD experiments show that anchor substitutions of GP2 do not greatly increase the stability. The best peptide is the double substitution I2L/L9V, but even it is deficient compared with ML. *E*, T2 cell surface stabilization confirms the CD data. *F*, the cell surface half-lives are moderately increased compared with GP2.

immunologically reactive peptides do not bind well to class I MHC molecules. If the peptides dissociate from class I MHC molecules too quickly, the cells presenting the peptides do not have a sufficient concentration of the specific pMHC at the surface of the cell to be recognized by circulating T cells. We examined binding of a selection of known immunologically recognized

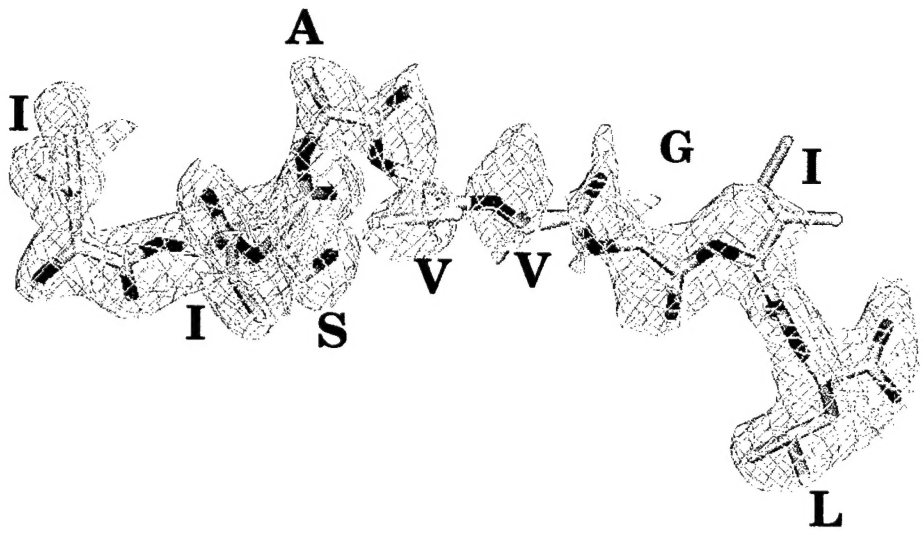


FIG. 2. **The center of the GP2 peptide is disordered.** The averaged omit electron density map of the GP2 peptide with a cover radius of 1.5 Å. The map was calculated using modified phases from DM.

peptide ligands from the tyrosine kinase family member HER-2/neu. Despite the presence of CTL that recognize these peptides bound to A2, the tumors are not eliminated. These HER-2/neu peptides displayed a spectrum of binding affinities, but all were lower than the level observed for high affinity binders, such as ML or RT. Of particular interest to the immunology of tumor recognition was the clustering of many of these peptides in the "low affinity" category. Remarkably, all of these peptides, (GP2, C84, and F56) have good anchor residues for A2.

There are two primary reasons to examine this phenomena in detail. The first is to understand how class I MHC binds peptides. There is a great deal known about how class I MHC binds many peptides with great sequence diversity, but there is very little information about how the protein binds any particular peptide well or poorly. There are now many examples of crystal structures of high affinity peptides bound to class I MHC. GP2 is a perfect example of a poor binding peptide and as such offers the first opportunity to understand poor binding. The second reason to examine GP2 is that poor affinity peptides are potentially better targets for immunotherapy. The rationale for this has to do with T cell education. T cells are selected for survival by two mechanisms (positive and negative selection) in the thymus (35). If a self-peptide binds to class I MHC with high affinity, there is a larger concentration of pMHC in the thymus and thus a greater chance that T cells would be able to recognize the complex well. Presumably, this set of T cells would be deleted from the T cell receptor repertoire, and they would not be in the periphery. If the self-peptide binds with poor affinity, the concentration of that peptide-MHC molecule in the thymus may be too low for recognition during the selection process. Therefore, there is a greater probability of finding these T cells in the periphery.

A complex of A2 with GP2 bound has poor thermal stability ($T_m$, 36.4 °C) and a very short cell surface half-life (~21 min). Many laboratories including ours have improved the binding affinity of peptides by changing the anchor residues to those most preferred by A2 (13, 36). As an example, we show that a small change in the anchor position of the melanoma peptide MelA results in a peptide with much greater binding affinity. However, trials with substitutions of GP2 at the anchor positions showed that the affinity was not significantly improved (Table I). Our goal is to be able to design APL for cancer immunotherapy. To be able to do this in a reasonable fashion, we needed to determine the crystallographic structure of A2-GP2 and determine why this peptide binds poorly.

The crystallographic structure shows uninterpretable electron density within the center of the peptide. Our interpretation of these data is that the peptide does not assume one unique conformation in the center as has been seen for all other single peptide-MHC structures to date (reviewed in Ref. 4). Interestingly, this is analogous to the situation found in the crystal structure of a class I MHC complex that contained a mixture of many different peptides (37). These data suggest that anchor substitutions do not significantly increase the affinity of GP2 because they do not address the fundamental problem that the peptide has in binding. The center does not make stabilizing contacts with the binding cleft of the class I MHC molecule.

This result begs another important immunological question. Does the flexibility in the center of the peptide increase or decrease immunogenicity? On the one hand, the flexibility decreases the already small concentration of a specific molecular surface that can interact with the T cell receptor on a circulating T cell. On the other hand, multiple peptide conformations generate more molecular surfaces that can be potentially recognized by circulating T cells. Perhaps in the context of a peptide that binds well, increased flexibility is more immunogenic, but in the context of a poor binding peptide increased flexibility does not increase immunogenicity because of the reduced concentration effect.

There is increasing interest in using peptides that bind to class I MHC for immunotherapy. As is the case of vaccination used to prevent viral infection, the potential therapeutic value is significant. As more antigens are discovered that are recognized by CTL and yet bind poorly to class I MHC molecules, the rules that predict binding affinity will be more critical. The phenomena observed here for GP2 certainly applies to other poor binding peptides whose binding affinity is not increased by altering the anchor residues. Increased affinity can be obtained for many of these peptides, but a full understanding of how peptides bind to class I MHC is still needed. By examining the binding of GP2 at the atomic level, we have made another step toward understanding peptide binding well enough to make predictions that will increase peptide affinity and minimize immunological consequences.

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